Clarification of contribution over the art

Following is further clarification regarding the contribution over the art of this application in response to the 9/15/06 Office communication, regarding the issue of the specificity of the primers used in claim 1, and the yield of expression product in claims 2 and 3.

State of the art

The state of the art is the method to obtain full-length recombinant SMN protein based on the isolation of SMN-cDNA clones from the cDNA library using the synthesized oligonucleotide probes which is very time-consuming and requires highly skilled personnel to perform.

Contribution over the art

The present application offers a new method to overcome the above mentioned technical problem. What is inventive and what a person of ordinary skill in the art would not do is to conceptualize a different approach a) to find the easiest and fastest method via the RT-PCR reactions to get the SMN constructs, and b) to select the most powerful expression vectors commercially available in order to get different powerful expression constructs. These expression constructs would thus allow full-length recombinant SMN protein to be obtained in different expression systems.

Reverse Transcription (RT) and Polymerase Chain Reaction (PCR) in claim 1

Issue of specificity of the primers stated in claim 1

For the purpose of research work in this field of molecular biology, the actual issue is whether one can obtain specificity or not. In this applicant's work, the specific

primers SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3 were used to perform the reverse transcription (RT) and polymerase chain reaction (PCR) from the total RNA. The contribution of the art is demonstrated through the actual result of clear and obvious specificity as shown by only one band, with high quantity of 0.9 kb of the PCR product corresponding to the CDS of the SMN gene obtained by electrophoresis analysis on a 20 g/L agarose gel (see attached copy of Figure 1 of the application). In the field of molecular biology, obtaining only one band, with high quantity of the PCR product is the real proof of the applicant's contribution over the art in terms of the specificity of the selected primers (SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3) and the efficacy of the optimized conditions for RT-PCR reactions as described in claim 1. Otherwise, if there is a lack of specificity from the selected RT-PCR primers stated in claim 1, the amplification of the CDS of the SMN gene cannot take place; in this case, there would be no band of 0.9 kb of the PCR products. A best situation due to lack of specificity of the selected RT-PCR primers would not result in the amplification of the CDS of the SMN gene alone but also the amplification of other different genes; in this case, other different bands of different sizes would be present, besides the band of 0.9 kb corresponding to the CDS of the SMN gene product.

Therefore, the applicant's contribution **over the art** is in:

-selecting the specific primer for RT reaction (SEQ ID NO:1) in order to get the specific SMN-cDNA containing the CDS of the SMN gene;

-selecting the specific primers (SEQ ID NOS: 2 and 3), and optimizing the PCR reaction conditions by taking into account the specific properties (base composition,

length and concentration) of the target gene and primer sequences in order to get the PCR product corresponding to the CDS of the SMN gene;

-determining the number of 35 cycles for PCR amplification in order to get high quantity of the PCR product.

Utilization of the commercially available expression vectors for the construction of the expression plasmids for full-length recombinant SMN protein in claims 2 and 3

Issue of expression products

The applicant's contribution over the art is in developing the easiest and fastest way which requires creating a new way to create the Bam HI and Xho I restriction sites on the obtained SMN-cDNA products by ligating them to the PCR II vector (SEQ ID NO. 4) commercially available in which the Bam HI and Xho I restriction sites are already present, in order to produce vector (**1**) (SEQ ID NO. 4 / SMN cDNA) – for elaboration of this process, refer to p. 17-19 in the 8/15/06 Response draft). This is crucial for the construction of the expression plasmids for SMN protein from the selected expression vectors in the next step:

Selecting the most powerful expression vectors commercially available that have Bam HI and Xho I restriction sites in their multiple cloning sites (Refer to p. 18B of the 8/15/06 Response draft), such as pFast Bact Htb (SEQ ID NO. 5), pBlue Bac His 2A (SEQ ID NO. 6), and pET-28a (+) (SEQ ID NO. 7) to get most easily and rapidly the different SMN constructs (**2**) SEQ ID NO. 5 / SMN-cDNA; (**4**) SEQ ID NO. 6/ SMN-cDNA; (**5**) SEQ ID NO. 7 / SMN-cDNA in order to rapidly produce a high yield of full-length recombinant SMN protein, capable of being purified via a simple, one step affinity-based purification process.

Due to time restrictions, the research work concerning the expression of the recombinant SMN protein from the expression constructs (**2**), (**4**), and (**5**) was not yet performed. However, the applicant has applied this same method in another research work to construct an expression plasmid for another protein, the Autographa californica nucleopolyhedrovirus DNA-binding protein (AcNPV DBP) in bacteria E. Coli cells from the expression plasmid pET-28 a (+) bearing the insert corresponding to the CDS of the AcNPV DBP gene: SEQ ID NO.7 / AcNPV DBP-cDNA. This expression construct is identical to that of (**5**) SEQ ID NO.7 / SMN-cDNA of the present application. The only difference is the gene.

Autographa californica nucleopolyhedrovirus (AcNPV) is a baculovirus widely employed as a vector for the expression of foreign genes and pest control. Although baculoviruses efficiently replicate in the nuclei of arthropod cells, the dynamics and mechanism of viral DNA replication within the infected cell are still poorly understood. Thus, to study such viral DNA replication, the availability of peptide ligands specific for AcNPV DBP is needed. This work entails the amplification of the CDS of the AcNPV DBP gene from AcNVP, the cloning of AcNPV DBP gene, the construction of the expression plasmid for AcNPV DBP, the expression of the recombinant His. Tag AcNPV DBP in E. Coli cells and the purification of the recombinant His. Tag AcNPV DBP which was used as a target molecule for the selection of the specific peptide ligands.

In this research work, the expression plasmid for AcNPV DBP was constructed using the pET-28a (+) (SEQ ID NO.7) as vector and resulting in the expression construct: SEQ ID NO.7 / AcNPV DBP-cDNA; this construction procedure is **the same as the one**

used to construct the expression plasmid (5) (SEQ ID NO.7 / SMN-cDNA) for recombinant SMN protein in the present application.

The results of expression of the recombinant His. Tag AcNPV DBP in bacteria BL21 (DE3) E. Coli cells from the expression construct SEQ ID NO.7 / AcNPV DBP-cDNA show that there is **rapid production of a large amount** up to 5 mg of recombinant His. Tag AcNPV DBP from 1 liter of bacteria culture after only 2 hours of expression using isopropyl-β-D-thiogalactopyranoside (I.P.T.G.) as inductor.

The obtained recombinant His. Tag AcNPV DBP was then **purified via a simple, one step affinity-based purification process** using the Ni-NTA His. Bind Resin column (Novagen, Madison, WI). This so obtained purified recombinant AcNPV DBP was used as a target molecule for the selection of the specific peptide ligands for AcNPV DBP which is valuable for diverse applications in basic as well as applied research.

The results of this research work were granted the U.S. Patent No. 7,101,966 and will be published in *Analytical Letters*, Vol. 40, Issue 02, 2007.

Over the art

Compared to 1 – 2 mg of recombinant protein obtained in bacteria using other expression vectors, what is over the art is the above mentioned results of 5 mg of recombinant His. Tag AcNPV DBP from 1 liter of bacteria culture obtained after only 2 hours of expression.

The data of this expression of the recombinant AcNPV DBP is a demonstration that the claimed methods in the present application provide an **easiest** and **fastest way to get a powerful expression construct** for obtaining recombinant protein in bacteria

which no one before has developed, and a person of ordinary skill in the art would not have done.

What is over the art is knowing how to select expression vectors in order to get powerful expression constructs: This applicant selected pET-28a (+) expression vector as the most powerful expression vector developed for the cloning and expression of recombinant proteins in bacteria because this expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that almost all of the cells resources are converted to target gene expression: The desired product can comprise more than 50% of the total cell protein in a few hours after induction by IPTG.

This system offers rapid, stable and high-yield expression of recombinant proteins modified with a histidine tag for simple and one-step affinity-based purification.

What is inventive and what a person of ordinary skill would not do is to **select the most powerful expression vectors** which is crucial to get an expression construct capable of rapidly producing a high yield of stable full-length recombinant protein. The powerfulness of the selected expression vectors is dependent on their own structures. The structure of the gene to be expressed does not matter; any gene can be expressed with a high yield by using the selected powerful expression vector and the appropriate expression system.

At the time of the submission of this application, there was no development of any powerful expression construct for full-length recombinant SMN protein. Therefore the contribution over the art is that this applicant has made available the different powerful expression constructs **(2)**, **(4)** and **(5)** for the purpose of rapidly producing large

amounts of full-length recombinant SMN protein in bacteria and in insect cells, capable of being purified via a simple, one-step affinity-based purification process.

The development of these expression constructs provides the users the choice to express full-length recombinant SMN protein in bacteria (mainly E. Coli cells) or baculovirus infected insect cells; the choice of the expression constructs and the corresponding expression systems should always be dictated by the goal to be reached. If large protein amounts are required for structural studies, heterologous expression using the expression construct (**5**) and E. Coli cells is advantageous because of its facility of use and its cost-effectiveness. If the goal is both structural and functional, using the expression constructs (**2**) or (**4**) and insect cells would be preferable.